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Regulation of the Rho Family Small GTPase Wrch-1/RhoU by C-Terminal Tyrosine Phosphorylation Requires Src▽†

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Wrch-1 is an atypical Rho family small GTPase with roles in migration, epithelial cell morphogenesis, osteoclastogenesis, and oncogenic transformation. Here, we observed rapid relocalization of Wrch-1 from the plasma membrane upon serum stimulation. Studies revealed a requirement for serum-stimulated tyrosine phosphorylation of Wrch-1 at residue Y254 within its C-terminal membrane targeting domain, mediated by the nonreceptor tyrosine kinase Src. Genetic or pharmacological loss of Src kinase activity blocked both phosphorylation and relocalization of Wrch-1. Functionally, Y254 was required for proper Wrch-1 modulation of cystogenesis in three-dimensional culture, and the phospho-deficient mutant, Y254F, was enhanced in Wrch-1-mediated anchorage-independent growth. Mechanistically, C-terminal tyrosine phosphorylation and subsequent relocalization of Wrch-1 downregulated its ability to interact with and activate its effectors by decreasing active Wrch-1-GTP, perhaps by altering proximity to a GEF or GAP. Phospho-deficient Wrch-1(Y254F) remained at the plasma membrane and GTP bound and continued to recruit and activate its effector PAK, even upon serum stimulation. In contrast, a phospho-mimetic mutant, Y254E, was constitutively endosomally localized and GDP bound and failed to recruit PAK unless mutated to be constitutively active/GAP insensitive. C-terminal tyrosine phosphorylation thus represents a new paradigm in posttranslational control of small GTPase localization, activation, and biological function.

Rho family proteins are Ras-related small GTPases that regulate cytoskeletal organization and dynamics, cell adhesion, motility, trafficking, proliferation, and survival (20). They function as tightly regulated molecular switches, cycling between an active GTP-bound state and an inactive GDP-bound state. Rho GTPases are also regulated by their subcellular localization, directed by sequences and posttranslational modifications, such as an isoprenoid lipid attached permanently to their C-terminal membrane targeting regions (1), and a second signal, such as a polybasic region or a palmitate fatty acid (34). Rho-guanine nucleotide dissociation inhibitors (RhoGDIs) bind prenyl groups and sequester Rho proteins from membranes (19, 42). Interaction of the GTP-bound proteins with their downstream effectors at specific locations then elicits their biological functions.

Wrch-1, also designated RhoU or Wrch1, is an atypical member of the Cdc42 subgroup of Rho GTPases that induce the formation of actin microspikes and filopodia. Although it shares 57% sequence identity with Cdc42 and 61% sequence identity with its closest relative, Chp/Wrch-2, Wrch-1 shares only partially overlapping localization and effector interactions with them and is regulated in a distinct manner. Like Cdc42, Wrch-1 activates PAK1 and JNK (13, 44), induces formation of filopodia (34, 35), and both morphological (8) and growth transformation in multiple cell types (5, 8). Wrch-1 also regulates focal adhesion turnover (13, 31), negatively regulates tight junction kinetics (8), plays a required role in epithelial morphogenesis (8), and modulates osteoclastogenesis (9, 10, 31).

Initially discovered as a Wnt-responsive gene capable of phenocopying Wnt morphological transformation (43, 44), Wrch-1 is transcriptionally regulated by Wnt (36), RANKL (10), and STAT3 (36), and it is upregulated in some cancers but downregulated in others (22). Thus, modulation of Wrch-1 activity at the level of expression is a common event. However, because it is a GTP-binding protein, a more dynamic regulation of Wrch-1 activity is also required.

Wrch-1 is thought to be largely GTP bound due to a high intrinsic exchange rate (2, 39), and no regulatory GEFs or GAPs have yet been identified. However, a putative dominant negative mutant of Wrch-1, T63N, does not behave like the wild type (34), so at least one GEF may be important to activate Wrch-1. Also, mutationally activated (Q107L, analogous to Q61L in Ras or Cdc42) Wrch-1 is more active than wild-type Wrch-1 (5, 8, 9, 31, 44), so one or more GAPs remain to be identified. Finally, Wrch-1 contains a negative regulatory 46-amino-acid N-terminal extension (39), and interaction with Grb2 or phospholipase Cγ1 (35, 39) may help to relieve autoinhibition (39).

In addition to these modes of regulation, Wrch-1 function requires posttranslational lipid modification of its C-terminal membrane targeting domain. Unusually, Wrch-1 is not preny-

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lated but is modified by palmitoylation (5), a dynamically regulated lipid modification (29) required for both its subcellular localization and biological activities (5, 8). Lacking a prenyl group, Wrch-1 does not bind RhoGDI (4). Both prenylation and the polybasic region of Cdc42 are required for its proper localization and function (46), but the identities of additional signals governing Wrch-1 are unknown.

There is increasing evidence that C-terminal serine/threonine phosphorylation of small GTPases near the isoprenoid moiety is required for both their localization and specific functions. In response to protein kinase C (PKC)-mediated phosphorylation of Ser181 in its C-terminal membrane targeting domain, K-Ras4B translocates from the plasma membrane to the mitochondria, where it then promotes apoptosis instead of proliferation (6). RalA is a target of Aurora A kinase-mediated phosphorylation at Ser194 (47) and PP2A Aβ-mediated dephosphorylation (7); phosphorylation of this site depletes RalA from the plasma membrane (27). Rap1 is phosphorylated on Ser180 by protein kinase A (26, 32), and RhoA localization and modulation of cell spreading and migration is regulated by PKA-mediated phosphorylation on Ser188 (17, 25), which promotes its binding to RhoGDI (17). PKCα (28, 33) and ROCK (33) stimulate phosphorylation of Rnd3/RhoE, which results in translocation, and PKC-mediated phosphorylation is required for Rnd3 to modulate the Rho/ROCK pathway (28). TC10 is phosphorylated by cyclin-dependent kinase 5 on Thr197, which regulates its association with lipid rafts, a requirement for its ability to modulate insulin-stimulated GLUT4 translocation (30). Thus, there is significant evidence for the functional importance of C-terminal serine/threonine modification of small GTPases.

Here, we sought to determine which C-terminal elements in addition to palmitoylation contribute to the regulation of Wrch-1 subcellular localization and biological activity. The minimal C-terminal membrane targeting sequence of Wrch-1 does not contain a suitable serine or threonine residue but contains a potentially phosphorylatable tyrosine residue. Further, subcellular localization is often dynamically regulated by external signals such as those supplied by serum factors that stimulate engagement of growth factor receptor tyrosine kinases and their associated nonreceptor tyrosine kinase partners (41). In this report, we describe our discovery that serum stimulates Src-mediated tyrosine phosphorylation of Wrch-1 and its translocation from the plasma membrane and that a specific tyrosine residue in the C-terminal membrane targeting domain of Wrch-1 regulates its subcellular localization, GTP/GDP-binding status, effector activation, and biological activities. Thus, we have identified C-terminal tyrosine phosphorylation as a novel mechanism for regulation of small GTPase activity.

MATERIALS AND METHODS

Molecular constructs. Mammalian expression constructs for green fluorescent protein (GFP)-tagged and hemagglutinin (HA) epitope-tagged human Wrch-1 (wild-type [WT] and Q107L) have been described previously (5). Phospho-deficient Wrch-1(Y254F) and phospho-mimetic Wrch-1(Y254E) were generated by site-directed mutagenesis in both WT and Q107L backgrounds. The GFP-fusion protein of the C-terminal 9 amino acids (the "9-aa tail") of Wrch-1 expressed from pEGFP has been described previously (5). An additional GFP fusion containing the C-terminal 19 amino acids (19-aa tail) was generated by site-directed mutagenesis in the same manner. All sequences were verified by the Genome Analysis Facility at UNC-CH. GFP-PAK-PBD and GST-PAK-PBD were kind gifts from Channing Der (UNC-CH) and Keith Burridge (UNC-CH), respectively. WT Src, constitutively active Src(Y528F), and kinase-deficient Src(K297R) were expressed from the pUSE vector, all from Upstate Biotechnology. Bacterial expression constructs and purification of GST-Wrch-1 protein have been described previously (38).

Cell culture, transfections, and retroviral infection. H1299 non-small cell lung cancer (NSCLC) cells were grown in Dulbecco's modified Eagle medium (high glucose; DMEM-H; GIBCO/Invitrogen) supplemented with 10% fetal bovine serum (FBS; Sigma) and 1% penicillin-streptomycin (P/S) ("complete culture medium") and maintained in 5% CO_2 at 37°C. H1299 cells were transfected with expression constructs encoding Wrch-1, Src, or PAK-PBD proteins by using TransIT-LT1 (Mirus) according to the manufacturer's instructions. For localization assays, cells were transfected transiently and used 24 h after transfection. For selection of stably expressing cell lines, cells were grown in complete medium supplemented with the appropriate antibiotic for 5 to 7 days, after which >50 colonies were pooled for use.

MDCKII cells, generously provided by Robert Nicholas (UNC-CH), were grown as above and supplemented with 1% nonessential amino acids (NEAA; Invitrogen) ("complete medium"). MDCKII cell lines stably expressing Wrch-1 were generated by retroviral infection. Retrovirus was collected following $CaCl_2$-mediated transfection of pBabe-HAII-puro, pVPack-Gag/Pol, and pVPack-Ampho (Stratagene) expression vectors into 293T cells. Cells were infected by exposure to retroviral supernant containing 8 μg/ml of Polybrene (American Bioanalytical) and maintained in puromycin for 10 days, after which the colonies were pooled for use.

SYF mouse embryo fibroblast cells (MEFs) genetically lacking Src, Yes, and Fyn, or YF cells lacking Yes and Fyn but retaining Src (23), were grown in complete culture medium as described above. Cells were transiently transfected with constructs encoding HA-Wrch-1 proteins by using TransIT-LT1 (Mirus) according to the manufacturer's instructions.

Fluorescence, immunofluorescence, confocal microscopy, and localization assays. Cells were transfected transiently with pEGFP-Wrch-1 expression vectors or pEGFP-PAK-PBD as indicated above and grown in complete medium for 24 h. The cells were then either grown further overnight in complete medium ("basal conditions"), or serum starved overnight in 0% serum ("serum starved"), or serum starved overnight in 0% serum and then stimulated for 2, 5, or 15 min with fresh serum-containing complete medium ("starved and stimulated"). For some experiments, cells were treated for 1 h with the Src family kinase inhibitor SU6656 (Sigma) or dimethyl sulfoxide vehicle prior to serum stimulation. Following incubation with Alexa 647-transferrin (Molecular Probes), cells were fixed and then visualized for GFP-Wrch-1 (green) or transferrin (red). HA-Wrch-1 was visualized by staining with a primary anti-HA antibody (Covance) and a secondary anti-mouse antibody conjugated to Alexa 647 (Invitrogen). Confocal microscopy was performed on an Olympus Fluoview 500 laser scanning confocal imaging system, configured with an IX81 fluorescence microscope fitted with a PlanApo 60× oil objective.

Antibodies and immunoblot analysis. Western blot analyses were carried out as described previously (8). Briefly, cells were lysed in magnesium lysis buffer (MLB) containing 1× protease inhibitor cocktail (Roche) with or without 100 μM pervanadate, lysates were cleared by centrifugation, and protein concentrations were determined using the DC Lowry protein assay (Bio-Rad). Twenty micrograms of protein for each sample, prepared in 5× Laemmli sample buffer, was resolved by using 12% SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes (PVDF; Millipore), blocked overnight in 3% fish gelatin, and then probed for Wrch-1 by a 1-h incubation with the following primary antibodies: anti-HA epitope (HA.11; Covance), antiphosphotyrosine (pY100 from Cell Signaling Technologies [CST] or pY20 from Santa Cruz Biotechnology), anti-GFP (JL8; Clontech), anti-PAK1/2/3 (CST), anti-phospho-PAK1(Thr423)/-PAK2(Thr402) (CST), anti-Pyk2 (50), or anti-phospho-Pyk2(Tyr402) (Biosource). Anti-β-actin (Sigma) was used to demonstrate equivalent loading. Washed membranes were incubated in anti-mouse or anti-rabbit IgG–horseradish peroxidase (HRP; Amersham Biosciences) or anti-mouse kappa light chain–HRP (Zymed), washed again, and developed using Super-Signal West Dura extended duration substrate (Pierce).

Immunoprecipitation. H1299 cells expressing GFP- or HA-tagged Wrch-1 were lysed in MLB with protease inhibitor, with or without pervanadate, as described above, at 24 h after transfection. Lysates were precleared with protein A/G beads (Santa Cruz) and then incubated overnight with anti-GFP or anti-HA antibody. After 18 h, the protein-antibody complexes were recovered using protein A/G beads. Beads were collected and washed with MLB and resuspended in Laemmli sample buffer, the precipitated proteins were resolved on

SDS-PAGE, and immunoblot analysis for phosphotyrosine was performed as described above.

***In vitro* tyrosine kinase assay.** Recombinant Wrch-1 protein was used as a substrate for purified Src kinase in a standard *in vitro* kinase assay. Bacterially expressed GST-Wrch or GST alone was incubated for 40 min at 30°C with or without 0.8 μg of purified recombinant Src protein (Upstate Biotechnology) in Src kinase reaction buffer (100 mM Tris [pH 7.2], 125 mM $MgCl_2$, 25 mM $MnCl_2$, 2 mM EGTA, 100 μM Na_3VO_4, and 2 mM dithiothreitol) containing [γ-^{32}P]ATP (10 μCi per reaction mixture). Reactions were terminated by the addition of 4× sample buffer and then heated at 95°C for 5 min. Protein samples were separated by 10% SDS-PAGE and visualized by Coomassie blue staining. Incorporation of radiolabel was determined by audioradiography.

Anchorage-independent growth transformation assay. Single-cell suspensions of MDCK cells (3.5×10^3 cells per 35-mm dish) were suspended in 0.4% agar (BD Biosciences) in complete medium and layered on top of 0.6% agar as described previously (8). After 14 days, colonies were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) after counting of small colonies (6 to 15 cell diameters) and large colonies (>15 cell diameters), and the average number of each type of colony on triplicate dishes was quantified. A one-way analysis of variance (ANOVA) and Tukey's post hoc test were performed; P values of <0.01 were considered significant.

Epithelial morphogenesis cyst formation assay. MDCKII cells stably expressing HA-tagged Wrch-1 proteins were allowed to form cysts in three-dimensional (3D) collagen matrices as described previously (8). Briefly, monodispersed MDCKII cells were allowed to grow and form multicellular cyst structures on collagen I gels for 10 days, when the cultures were treated with collagenase type VII (C-2399; Sigma). Cyst structures were fixed and permeabilized, then incubated with fluorescent Texas Red-phalloidin (Molecular Probes) and mounted for imaging on an Olympus Fluoview confocal microscope as indicated above. Multiple *x-y* and *x-z* scans were acquired for each 3D collagen gel. We have shown previously that tightly regulated endogenous Wrch-1 activity is critical for proper cystogenesis on 3D collagen I matrices in these cells (8). In each of three replicate experiments, 25 multicellular cyst structures were evaluated for each Wrch-1-expressing cell line and binned into one of the following three groups: normal cysts (cysts containing a single lumen) or one of two groups of abnormal cysts (either cysts containing no lumen or cysts containing multiple lumens), as we have done previously (8). Student's t test was performed to determine significance; P values of <0.01 were considered significant.

Wrch-1 activation assay. H1299 cells were transiently transfected with either pCGN vector only or pCGN vectors encoding HA-Wrch-1, HA-Wrch-1(Y254F), HA-Wrch-1(Y254E), HA-Wrch-1(Q107L), HA-Wrch-1(Q107L/Y254F), or HA-Wrch-1(Q107L/Y254E), by using TransIT LT1 as described above. Cells were serum starved overnight, or serum starved and then serum stimulated for 2, 5, or 15 min as described above. Cells were then washed twice with ice-cold phosphate-buffered saline (pH 7.4) and lysed in MLB as described above. Equal volumes were removed from each lysate for total protein analysis. To each lysate, glutathione-agarose beads containing 40 μg of GST-p21-activated kinase (PAK) GTPase-binding domain fusion protein (GST-PAK-PBD) were added and incubated at 4°C for 60 min with rocking. Agarose-GST-PAK-PBD and associated Wrch-1 was pelleted and washed three times with 500 μl wash buffer (25 mmol/liter Tris [pH 7.5], 40 mmol/liter sodium chloride, and 30 mmol/liter magnesium chloride). Final pellets were resuspended in 1× protein sample buffer and resolved on SDS-PAGE. HA-Wrch-1 was detected using anti-HA antibody (Covance).

RESULTS

Wrch-1 rapidly relocalizes from the plasma membrane in response to serum stimulation. We have shown previously that the atypical Rho family small GTPase Wrch-1 localizes to both plasma membrane and internal compartments, including endosomal membranes (5). Because localization of other proteins, including some Rho GTPases, can be directed by external stimuli, such as serum-stimulated engagement of growth factor receptors, and is therefore dynamically regulated, we sought to determine whether Wrch-1 localization was similarly regulated. We serum starved overnight H1299 NSCLC cells transiently expressing enhanced GFP (EGFP)-tagged Wrch-1 (designated GFP-Wrch-1) and stimulated them with serum for 15 min. Under basal or serum-starved conditions (Fig. 1, left and middle columns), Wrch-1 was localized both to the plasma membrane and to endosomal membranes (marked by transferrin; middle row). In contrast, upon serum stimulation, Wrch-1 underwent rapid loss from the plasma membrane (Fig. 1, right column) but continued to localize to endosomes. Time-lapse video taken over the same time course confirmed relocalization of Wrch-1 from the plasma membrane (see Movie S1 in the supplemental material). These data indicate that Wrch-1 subcellular localization is both dynamic and regulated by upstream growth signals.

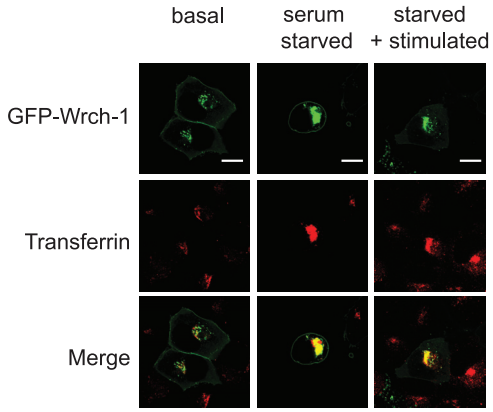

FIG. 1. Wrch-1 rapidly relocalizes upon serum stimulation. H1299 NSCLC cells transiently transfected to express GFP-Wrch-1 were grown in complete culture medium, then serum starved overnight, or first serum starved and then serum stimulated. Prior to serum stimulation, the treated cells were incubated for 1 h with AlexaFluor 647-labeled transferrin to mark endosomal compartments. After 15 min of serum stimulation, cells were fixed and then subjected to confocal microscopy for visualization of GFP-Wrch-1 (green) or transferrin (red). Bars, 20 μm.

Relocalization is dependent on the presence of a tyrosine at position 254 in the Wrch-1 C-terminal membrane targeting domain. We had previously determined that the carboxy-terminal 9 amino acids of Wrch-1 are sufficient for its proper subcellular localization (5) and that mutation of the palmitoylated cysteine residues therein results in cytosolic accumulation (5). Within this sequence of WWKKYCCFV, the single tyrosine at residue 254 (Y254) stood out. We hypothesized that Y254 becomes tyrosine phosphorylated in response to serum stimulation and that this phosphoryation event modulates Wrch-1 subcellular localization and activity.

We therefore generated a Y→F mutation at position 254 [designated Wrch-1(Y254F)]. The putatively phospho-deficient Wrch-1(Y254F) was resistant to serum-stimulated relocalization: the plasma membrane-localized pool of Wrch-1(Y254F) remained on the plasma membrane even after serum stimulation (Fig. 2A; see also Movie S2 in the supplemental material). Further, cells expressing this nonphosphorylatable mutant displayed an exaggeratedly rounded phenotype under basal serum-containing conditions, similar to that seen in serum-starved cells expressing only WT Wrch-1 (Fig. 2A) and consistent with a serum-dependent event altering both Wrch-1 phosphorylation and cytoskeletal organization.

These results were also consistent with our hypothesis that Wrch-1 becomes tyrosine phosphorylated on Y254 in response to serum stimulation and that this phosphorylation event is

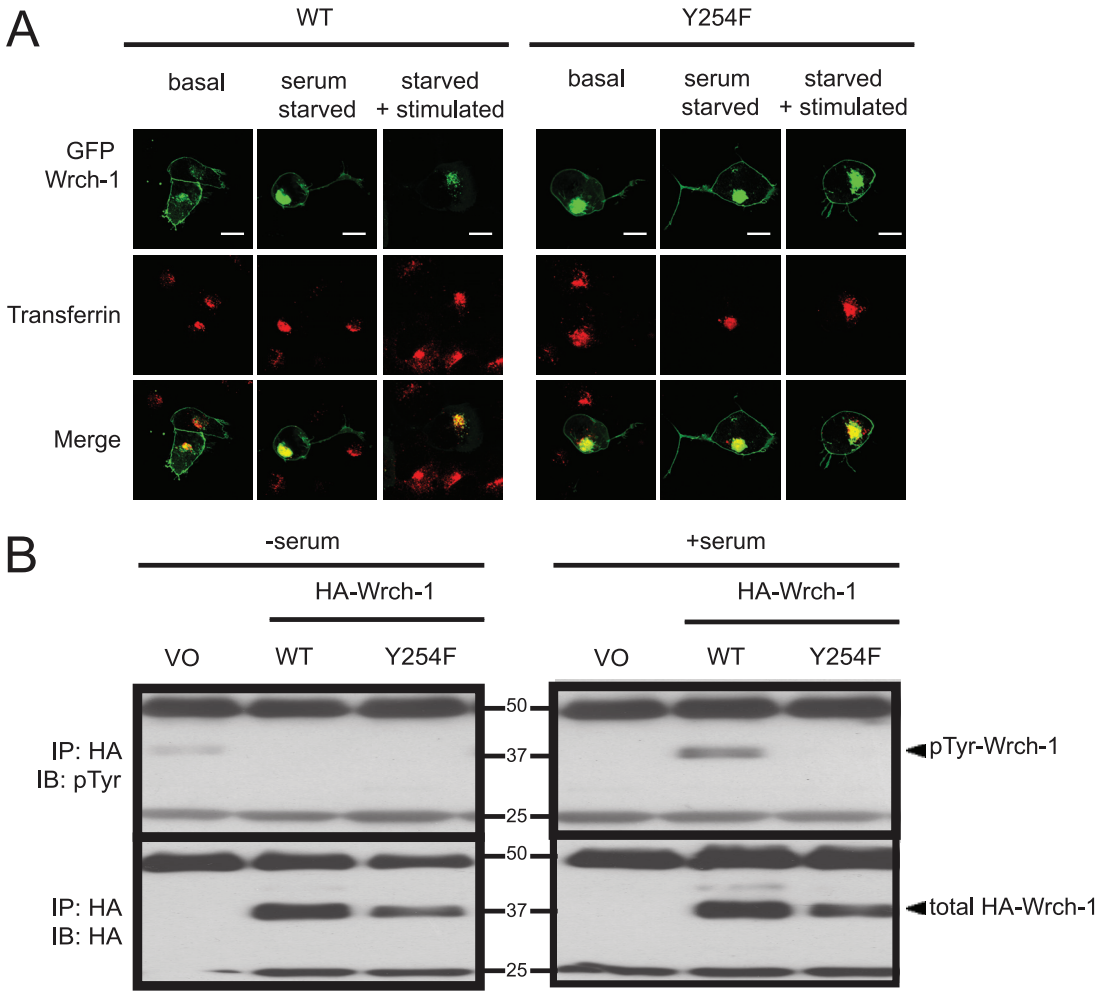

FIG. 2. Wrch-1 is tyrosine phosphorylated on Y254 in response to serum, and this phosphorylation is required for serum-stimulated relocalization. (A) Nonphosphorylatable Wrch-1(Y254F) is resistant to serum-stimulated relocalization. H1299 cells expressing either GFP-Wrch-1 or GFP-Wrch-1(Y254F) were grown, treated, and evaluated as described for Fig. 1. Bars, 20 μm. (B) Serum-stimulated tyrosine phosphorylation of Y254. H1299 cell lysates from cells expressing empty vector (VO), HA-Wrch-1, or HA-Wrch-1(Y254F) were incubated with anti-HA antibody. Immunoprecipitated (IP) Wrch-1 was detected by immunoblotting (IB) with anti-HA, and phosphotyrosine (p-Tyr) on Wrch-1 was detected by immunoblotting with antiphosphotyrosine antibody. The bands above and below the Wrch-1 band represent immunoglobulin heavy chain and light chains, respectively. Apparent molecular masses are shown (in kilodaltons).

responsible for serum-stimulated relocalization of Wrch-1. To confirm that Wrch-1 was tyrosine phosphorylated at Y254, we immunoprecipitated Wrch-1 proteins from H1299 cells with anti-HA antibody and probed for phosphotyrosine. Wrch-1 but not Wrch-1(Y254F) was detected by anti-phosphotyrosine antibody following serum stimulation (Fig. 2B), confirming that Wrch-1 is tyrosine phosphorylated and suggesting that Y254 is the major site of serum-stimulated phosphorylation.

Interestingly, we also determined that basal subcellular localization of Wrch-1 requires less targeting information than does either tyrosine phosphorylation or serum-stimulated relocalization. The last 9 amino acids in the C terminus of Wrch-1 are sufficient for proper basal localization of Wrch-1 (5). Surprisingly, this short sequence (the 9-aa tail) (Fig. 3A) was not sufficient to support either tyrosine phosphorylation (Fig. 3B) or serum-stimulated relocalization (Fig. 3C). We speculated that 9 amino acids was an insufficient length to allow binding of the kinase. We generated an additional GFP-fusion protein comprising 19 amino acids of the Wrch-1 C terminus (the 19-aa tail), which still contained only a single tyrosine residue (Fig. 3A), which was sufficient both for tyrosine phosphorylation (Fig. 3B) and for relocalization (Fig. 3C). Interestingly, expression of the 19-aa tail resulted in altered cell size and shape, suggesting that it may sequester proteins important for regulating the actin cytoskeleton. Together, these data indicate that relocalization of Wrch-1 requires additional sequences compared to basal localization, perhaps to allow efficient kinase binding to the region encompassing Y254.

Src can phosphorylate Wrch-1, and Src tyrosine kinase activity is required for both tyrosine phosphorylation and serum-stimulated relocalization of Wrch-1. The nonreceptor tyrosine kinase Src, which transmits signaling from several serum-responsive growth factor receptor tyrosine kinases, has many substrates, not all of which have been defined clearly. We speculated that Src could phosphorylate Wrch-1 on Y254, and indeed we found that the Src family kinase inhibitor SU6656

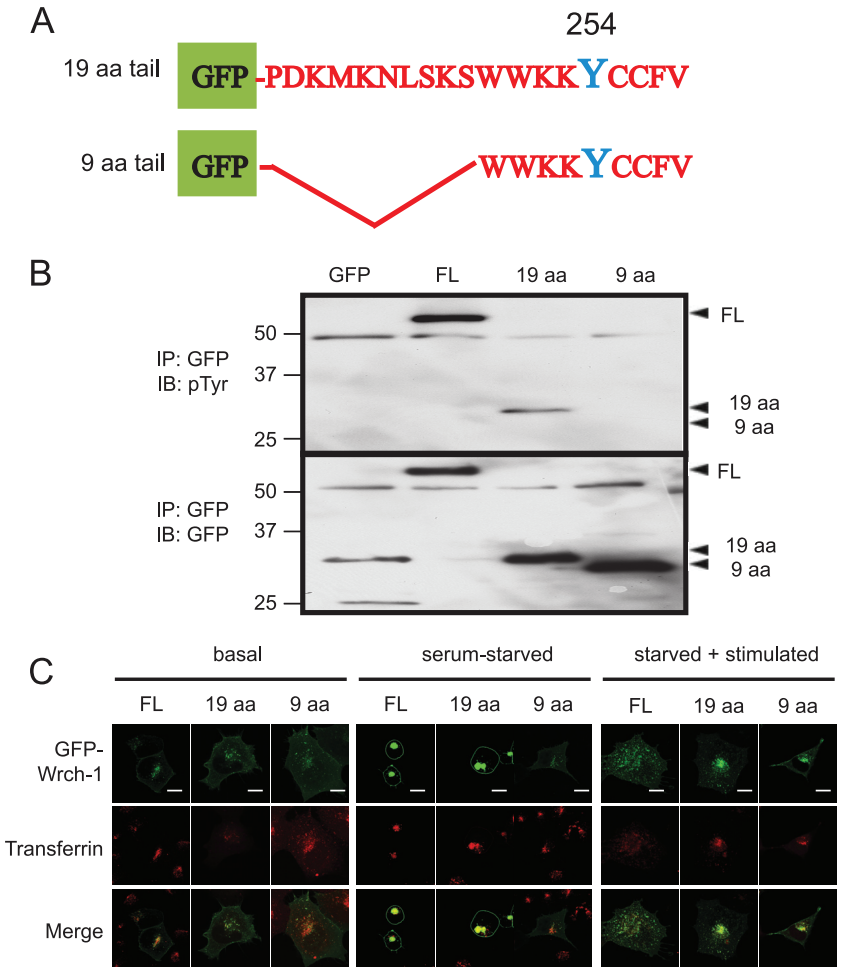

FIG. 3. The C-terminal 19 amino acids of Wrch-1 are sufficient for Wrch-1 to become tyrosine phosphorylated and to be relocalized in response to serum. (A) Schematic of the 19-aa and 9-aa tails. Shown are the GFP-tagged sequences extended with 9 or 19 amino acids of the C terminus of Wrch-1. Y254 is the only tyrosine residue present in each fusion protein. (B) Serum-stimulated tyrosine phosphorylation of the C-terminal 19 but not 9 amino acids of Wrch-1. H1299 cell lysates expressing either empty vector (GFP), GFP-Wrch-1 (FL), GFP fused to the 19-aa tail of Wrch-1, or GFP fused to the 9-aa tail ofWrch-1 were incubated with anti-GFP antibody to immunoprecipitate Wrch-1. Immunoprecipitated (IP) Wrch-1 was then detected by immunoblotting (IB) with anti-GFP antibody, and phosphotyrosine Wrch-1 was detected by immunoblotting with anti-phosphotyrosine antibody. (C) The C-terminal 19 amino acids of Wrch-1 are sufficient for serum-stimulated relocalization. H1299 cells as in panel B were grown, treated, and evaluated as described for Fig. 1. Bars, 20 μm.

effectively blocked tyrosine phosphorylation of Wrch-1 (Fig. 4A). At the concentration that we used (5 μM), SU6656 is reported to inhibit the related tyrosine kinases Src, Yes, Fyn, and Lyn (3). Therefore, we analyzed Wrch-1 phosphorylation in MEF cells from mice genetically deficient in Src, Yes, and Fyn (SYF; $Src^{-/-}$) (Lyn is restricted to hematopoietic cells) or deficient in Yes and Fyn but not Src (YF; $Src^{+/+}$). Wrch-1 was tyrosine phosphorylated in YF MEFs expressing Src but not in SYF MEFs lacking Src, indicating that Src is required for tyrosine phosphorylation of Wrch-1 (Fig. 4B). These data indicate that Src functions upstream of Wrch-1 to mediate its tyrosine phosphorylation. To address whether Src kinase enzymatic activity is required, we cotransfected H1299 cells with HA-Wrch-1 or HA-Wrch-1(Y254F) along with either constitutively active or kinase-deficient Src. Wrch-1 was tyrosine phosphorylated in the presence of kinase-active Src(Y528F) but not kinase-deficient Src(K297R), and the Y254 residue of Wrch-1 was required for this phosphorylation, as Wrch-1(Y254F) was not phosphorylated regardless of Src kinase activity (Fig. 4C). These data support a requirement for Src kinase activity in order for Wrch-1 to become tyrosine phosphorylated on Y254. To address whether the phosphorylation is direct or indirect, we performed an *in vitro* kinase assay with recombinant purified Src and GST-Wrch-1. Wrch-1 was directly phosphorylated by Src *in vitro* (Fig. 4D), consistent with the possibility that it may be phosphorylated directly *in vivo*.

Having established that Wrch-1 could be a substrate of Src kinase activity, and that the Y254 residue of Wrch-1 that is required for serum-stimulated relocalization is also the major site of Src-stimulated phosphorylation, we wished to test whether Src kinase activity is required for serum-stimulated relocalization of Wrch-1. We serum-starved cells expressing GFP-Wrch-1, treated them with SU6656 (5 μM) for 1 h, then serum-stimulated them as above. SU6656 prevented Wrch-1 relocalization in response to serum stimulation (Fig. 4E). Collectively, these results indicate that Src tyrosine kinase activity

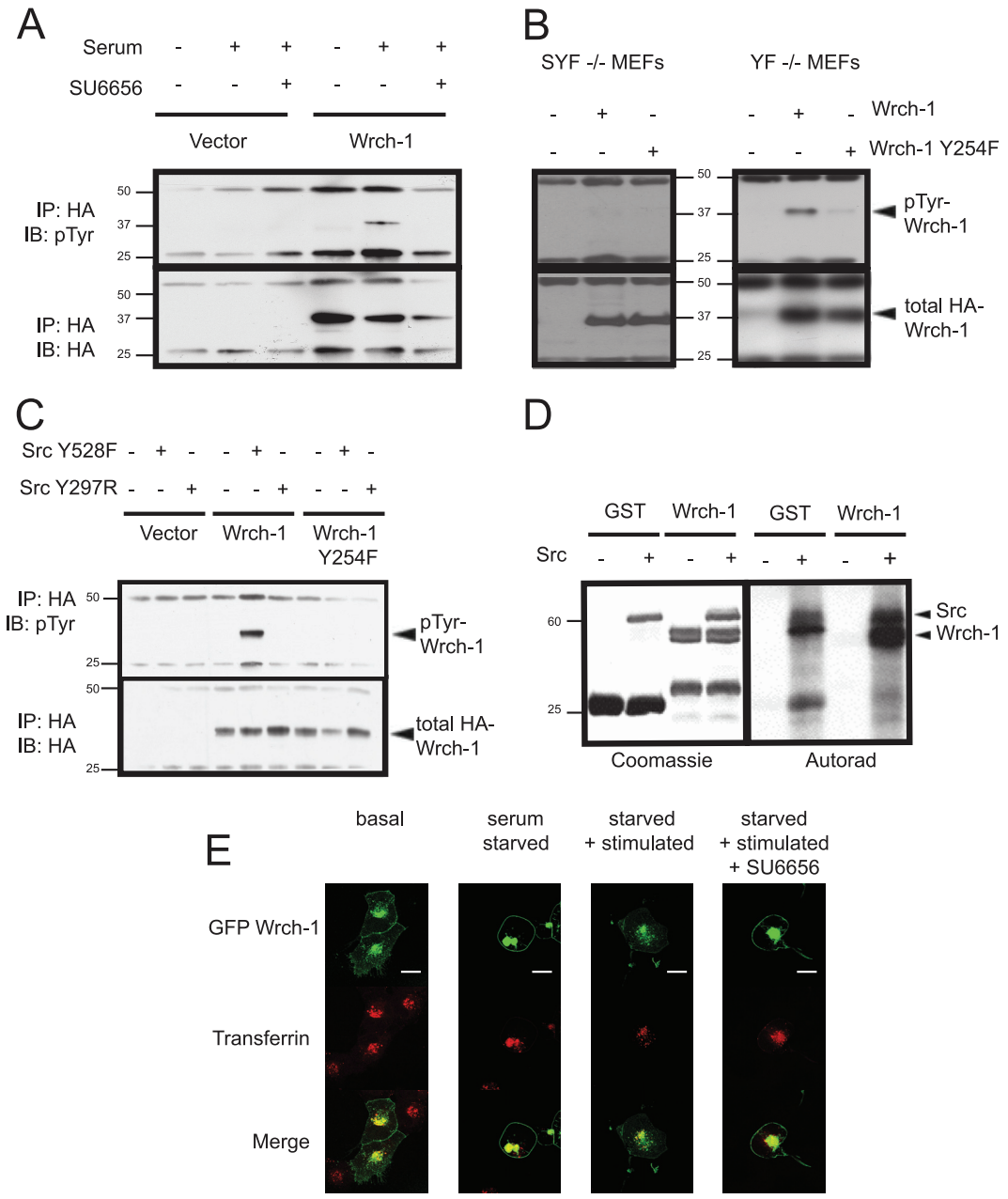

FIG. 4. Src activity is required *in vivo* for tyrosine phosphorylation of Wrch-1. (A) The Src family tyrosine kinase inhibitor SU6656 prevents Wrch-1 tyrosine phosphorylation in response to serum stimulation. H1299 cells expressing HA-Wrch-1 were serum starved overnight, treated with 5 μM SU6656 for 1 h, and serum stimulated for 5 min. Lysates were subjected to immunoprecipitation (IP) with anti-HA to retrieve HA-Wrch-1, followed by immunoblotting (IB) for Wrch-1 (anti-HA) or phosphotyrosine (anti-p-Tyr). Bands above and below the Wrch-1 band represent the Ig heavy chain and light chain, respectively. (B) Endogenous Src is required for serum-stimulated Wrch-1 tyrosine phosphorylation. $SYF^{-/-}$ MEFs (MEFs lacking Src, Yes, and Fyn) and $YF^{-/-}$ MEFs (MEFs retaining Src but lacking Yes and Fyn) expressing either HA-Wrch-1 or nonphosphorylatable HA-Wrch-1(Y254F) were starved overnight and then serum stimulated for 5 min. The resulting cell lysates were probed for phosphotyrosine on Wrch-1 as described for panel A. (C) Src kinase activity is required for tyrosine phosphorylation of Wrch-1. H1299 cells were cotransfected with empty vector, HA-Wrch-1, or nonphosphorylatable HA-Wrch-1(Y254F) along with either empty vector, kinase-active Src (Src Y528F), or kinase-deficient Src (Src K297R). Phosphotyrosine on Wrch-1 was detected as shown in panel A. (D) Src directly phosphorylates Wrch-1 *in vitro*. Purified recombinant GST-Wrch-1 protein was incubated with purified recombinant Src tyrosine kinase protein and [^{32}P]ATP. Total protein was detected by Coomassie blue staining, and [^{32}P]ATP incorporation was detected by autoradiography. (E) Inhibition of Src kinase with SU6656 blocks serum-stimulated relocalization of Wrch-1. H1299 cells expressing GFP-Wrch-1 or nonphosphorylatable GFP-Wrch-1(Y254F) were grown in complete culture medium (basal) and then either serum starved overnight (serum starved) or first serum starved and then serum stimulated for 15 min, with or without 1 h of pretreatment with 5 μM SU6656. Cells were visualized as for Fig. 1 for Wrch-1 (green) or transferrin (red). Bars, 20 μm.

is required for both the tyrosine phosphorylation and relocalization of Wrch-1. Whether Src is a direct or indirect mediator of Wrch-1 phosphorylation *in vivo* remains uncertain.

Phosphorylatable residue Y254 regulates Wrch-1-mediated transformation. Correct subcellular localization is critical to regulate the biological functions of Rho GTPases (18). We hypothesized that tyrosine phosphorylation, which alters Wrch-1 subcellular localization, also likely alters at least some of its biological functions. Both wild-type and constitutively active Wrch-1(107L) are capable of inducing anchorage-independent growth transformation, such as colony formation in soft agar (5, 8), and endogenous Wrch-1 is required for correct epithelial cell morphogenesis (8). A nonpalmitoylated mutant of Wrch-1 that fails to localize correctly to the plasma membrane is unable to modulate either of these biological functions (5, 8). Therefore, we generated polarizable MDCKII epithelial cell lines stably expressing either HA-Wrch-1 or phospho-deficient HA-Wrch-1(Y254F) in both the WT and 107L backgrounds and confirmed that they expressed equivalent amounts of each protein (Fig. 5A, bottom panel). We then quantitated soft agar colony formation after 14 days of anchorage-independent growth. Cells expressing either of the nonphosphorylatable Y254F mutants formed a significantly increased number of large colonies (>15 cells in diameter) in soft agar compared to phosphorylatable Wrch-1 (Fig. 5A). These results indicate that tyrosine phosphorylation of Y254 represents a negative regulatory control of Wrch-1 biological activity.

Phosphorylatable residue Y254 regulates Wrch-1-mediated epithelial morphogenesis. Several Rho family small GTPases are required for cystogenesis in a 3D environment (11). In particular, Cdc42 is thought to be a master regulator of lumen development in tube and cyst structures, by virtue of its ability to regulate the Par6/atypical PKC (aPKC) polarity complex (11). Wrch-1 also binds Par6/aPKC, and a precise balance of endogenous Wrch-1 is also required for proper cystogenesis in a 3D environment, because either too little or too much Wrch-1 causes disruption of the formation of normal, single-lumen-containing, hollow cyst structures (8), and Cdc42 is unable to compensate for loss of Wrch-1. We therefore examined the effect of Wrch-1 tyrosine phosphorylation on this crucial Wrch-1-mediated function. MDCKII cells expressing phosphorylatable or phospho-deficient (Y254F) Wrch-1 were seeded in a matrix of collagen I, and cyst structures formed after 10 days were imaged with Texas Red-phalloidin. Constitutively phospho-deficient Wrch-1(Y254F) disrupted epithelial cell morphogenesis, as shown by a greatly decreased proportion of normal cysts containing a single lumen (Fig. 5B), and this disruption was greater than the disruption induced by phosphorylatable Wrch-1, whether in a WT or in a constitutively GTP-bound and active (107L) background (Fig. 5B). Together, these data indicate a requirement for the Y254 residue for proper function of at least two important biological activities of Wrch-1 and suggest that tyrosine phosphorylation at this site may be an important mechanism to precisely control Wrch-1 activity.

Wrch-1 interacts with its effector PAK at the plasma membrane, where it is GTP bound and active, but not at endosomes, where it is GDP bound and inactive. The lower biological activity displayed by wild-type Wrch-1 compared to the phospho-deficient Wrch-1 mutant Y254F that remains on the plasma membrane suggested that Wrch-1 is less able to interact with its effectors when it is endosomally localized than when it is localized to the plasma membrane. We therefore evaluated the ability of Wrch-1 to interact with its best-validated effector, PAK, at each of these locations, by utilizing a GFP-tagged form of the GTPase-binding domain of PAK (GFP-PAK-PBD). To test whether the same conditions of serum stimulation that cause Wrch-1 to become phosphorylated at Y254 and to relocalize away from the plasma membrane also decreased its interactions with PAK-PBD, we cotransfected H1299 cells with GFP-PAK-PBD and HA-tagged Wrch-1 or vector and observed their localization under conditions of serum starvation or stimulation. Representative images are shown in Fig. 6A, and the distribution of GFP-PAK-PBD primary localization to cytosol, plasma membrane, or endomembrane compartments under each condition was quantitated as depicted in Fig. 6B. As expected, in cells expressing only empty vector, GFP-PAK-PBD was distributed diffusely throughout the cell (Fig. 6A, green, and 6B, top left panel.) In contrast, in cells expressing HA-Wrch-1, GFP-PAK-PBD was recruited to specific membrane sites in a serum-dependent manner. In serum-starved cells, Wrch-1 was localized to both plasma membrane and internal membranes, as expected (Fig. 6A, red), whereas PAK-PBD (green) displayed overlapping localization with Wrch-1 (merged image; yellow) only at the plasma membrane. These results indicate that only the plasma membrane-localized pool, but not the internally localized pool, of Wrch-1 was able to recruit PAK-PBD (localized to plasma membrane in 78% of cells) (Fig. 6B). Upon serum stimulation, Wrch-1 redistributed away from the plasma membrane in a time-dependent manner as seen previously, such that by 15 min it was absent from the plasma membrane and localized predominantly at internal membranes. Under these conditions, PAK-PBD was not recruited to specific sites but was again distributed diffusely throughout the cytosol (83%), consistent with our hypothesis that tyrosine phosphorylation and relocalization of Wrch-1 from the plasma membrane decrease its ability to interact with and recruit effectors.

Because PAK-PBD binds preferentially to the active, GTP-bound form of Wrch-1, these results indicate that Wrch-1 is active at the plasma membrane but not at internal membranes. To confirm that Wrch-1 would still be able to bind PAK-PBD at internal membranes if it were active and GTP bound there, we examined the ability of a constitutively activated, GAP-insensitive mutant of Wrch-1 [Wrch-1(Q107L)] to recruit PAK-PBD. As expected, the 107L mutant still relocalized upon serum stimulation and still recruited PAK-PBD (Fig. 6A), even to internal membranes (85%) (Fig. 6B). Thus, the inability of WT Wrch-1 to recruit PAK-PBD following serum stimulation is not because PAK-PBD is simply unable to access endosomal compartments or to interact with tyrosine-phosphorylated Wrch-1.

Our observation that Wrch-1 recruited PAK-PBD to the plasma membrane but not to internal membranes further supports our hypothesis that tyrosine phosphorylation at Y254 and subsequent loss from the plasma membrane downregulates Wrch-1 activity. To further test this possibility, we examined recruitment of PAK-PBD by the nonphosphorylatable mutant

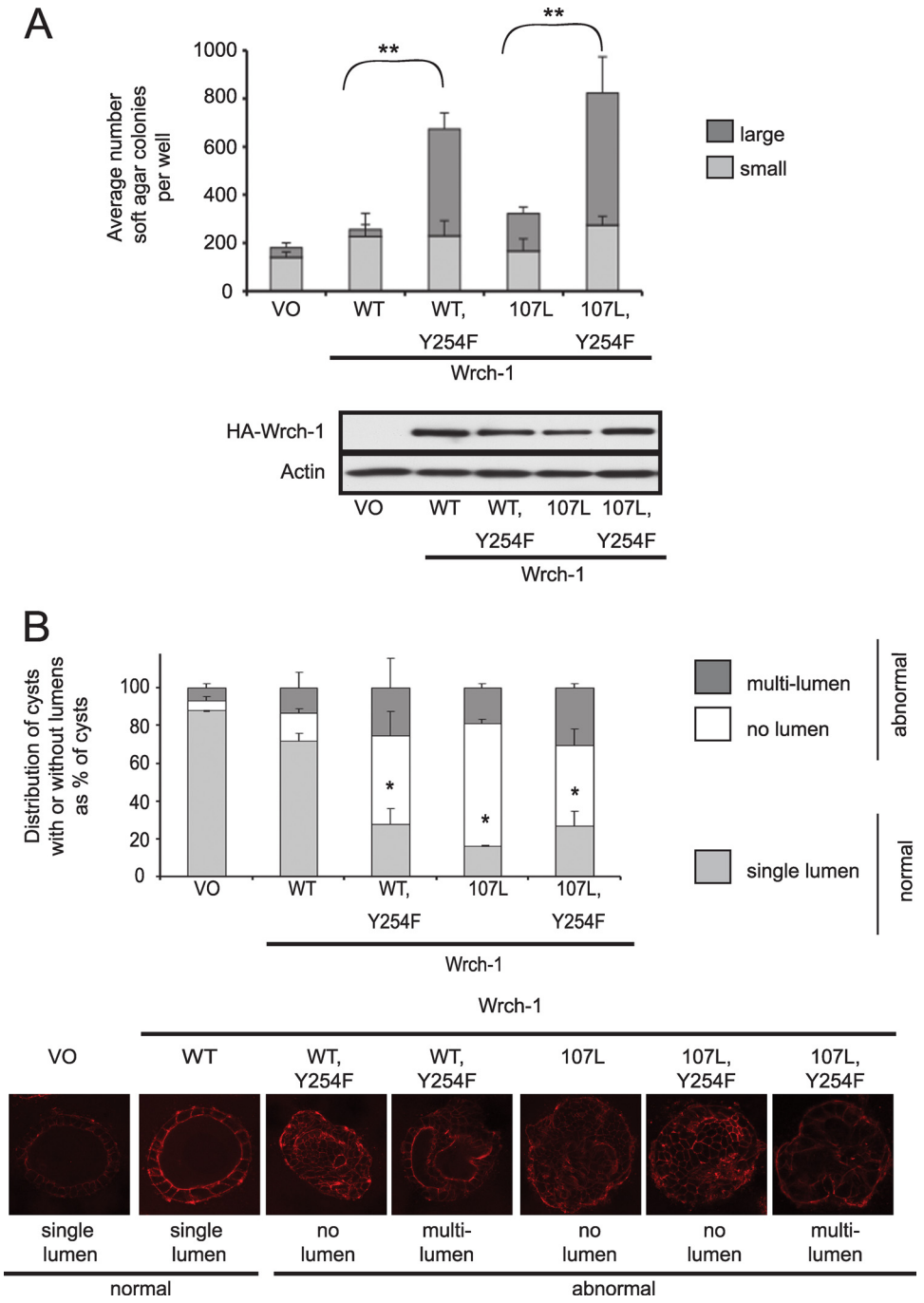

FIG. 5. Phosphorylation at Y254 negatively regulates Wrch-1-mediated biological functions. (A) Phosphorylatable tyrosine residue Y254 negatively regulates anchorage-independent growth. MDCKII cell lines were generated to stably express Wrch-1 proteins as indicated. Equivalent expression was confirmed by immunoblotting with anti-HA. β-Actin was used as a loading control. Cells were seeded into soft agar for anchorage-independent colony formation and grown for 14 days, then stained with MTT. Small colonies (6 to 15 cell diameters across) and large colonies (>15 cell diameters across) were quantified. Bar graphs indicate three independent experiments carried out in triplicate. One-way ANOVA and Tukey's *post hoc* tests were used to determine the significance of differences between numbers of colonies arising from cells expressing Wrch-1 with or without the Y254F mutation, in either the WT or constitutively activated (107L) backgrounds. **, $P < 0.001$. (B) Phosphorylatable tyrosine residue Y254 negatively regulates epithelial cell morphogenesis. MDCKII cells expressing Wrch-1 as in panel A were seeded into 3D collagen matrices and allowed to form cysts for 10 days. Cyst structures were evaluated and quantified according to whether they contained one lumen (normal; single lumen), more than one lumenal area (multilumen), or no lumen at all. Bars are the averages of three independent experiments for each cell line, with standard deviations. The significance of differences between numbers of normal cysts with single lumens in cells of different backgrounds was determined as described for panel A. *, $P < 0.01$.

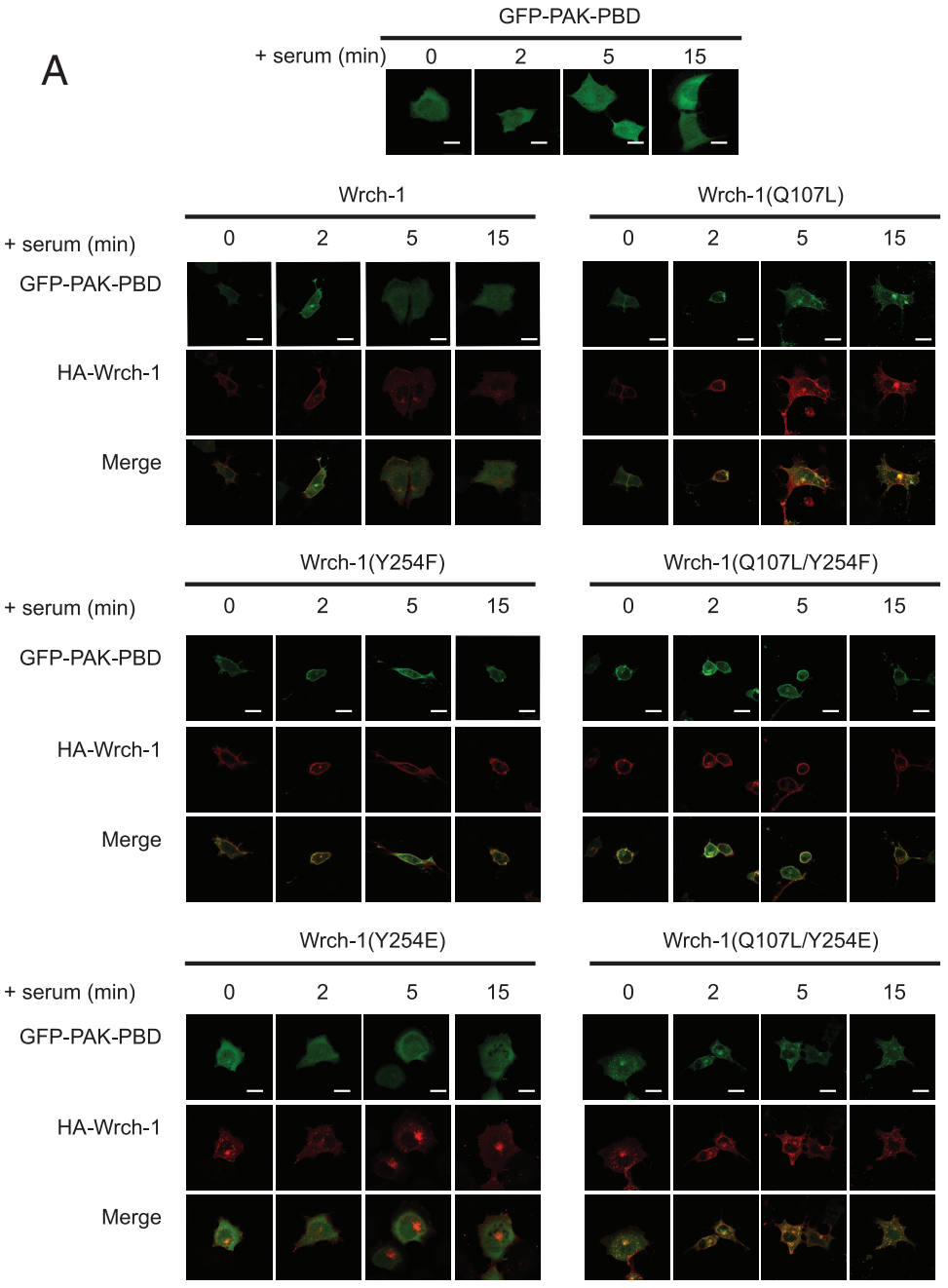

FIG. 6. Wrch-1 is GTP bound and active and recruits its effector PAK1 at the plasma membrane but not at endosomes. (A) Serum stimulation decreases recruitment of GFP-PAK-PBD to the plasma membrane by WT but not phospho-deficient Y254F Wrch-1. H1299 cells coexpressing GFP-PAK-PBD along with vector or Wrch-1 proteins were grown, treated, and evaluated as described for Fig. 1, except images were taken at multiple time points as shown. GFP-PAK-PBD signal is green and HA-Wrch-1 is red. Overlapping localization (merge; yellow) demonstrates recruitment of GFP-PAK-PBD by Wrch-1. Bars, 20 μm. (B) Quantitation of GFP-PAK-PBD subcellular distribution before and after serum stimulation. For each condition depicted with representative images in panel A, 50 cells from each of three independent experiments were evaluated for their subcellular distribution of GFP-PAK-PBD and quantified according to whether GFP-PAK-PBD localized primarily to the cytosol, the plasma membrane, or endosomal compartments. Results are presented graphically as the percentage of total cells counted in which the primary localization of GFP-PAK-PBD was identified as the specific compartment indicated. Bars represent the averages of three independent experiments for each cell line, with standard deviations.

Wrch-1(Y254F), in which the plasma membrane-bound pool remains on the plasma membrane and fails to relocalize in response to serum. As expected, the Y254F mutant continued to recruit PAK-PBD to the plasma membrane after serum stimulation (Fig. 6B, middle left panel; 84% plasma membrane localization) even in the absence of a concurrent constitutively activating mutation 107L (Fig. 6A). This result indicates that retention of a pool of Y254F at the plasma membrane is

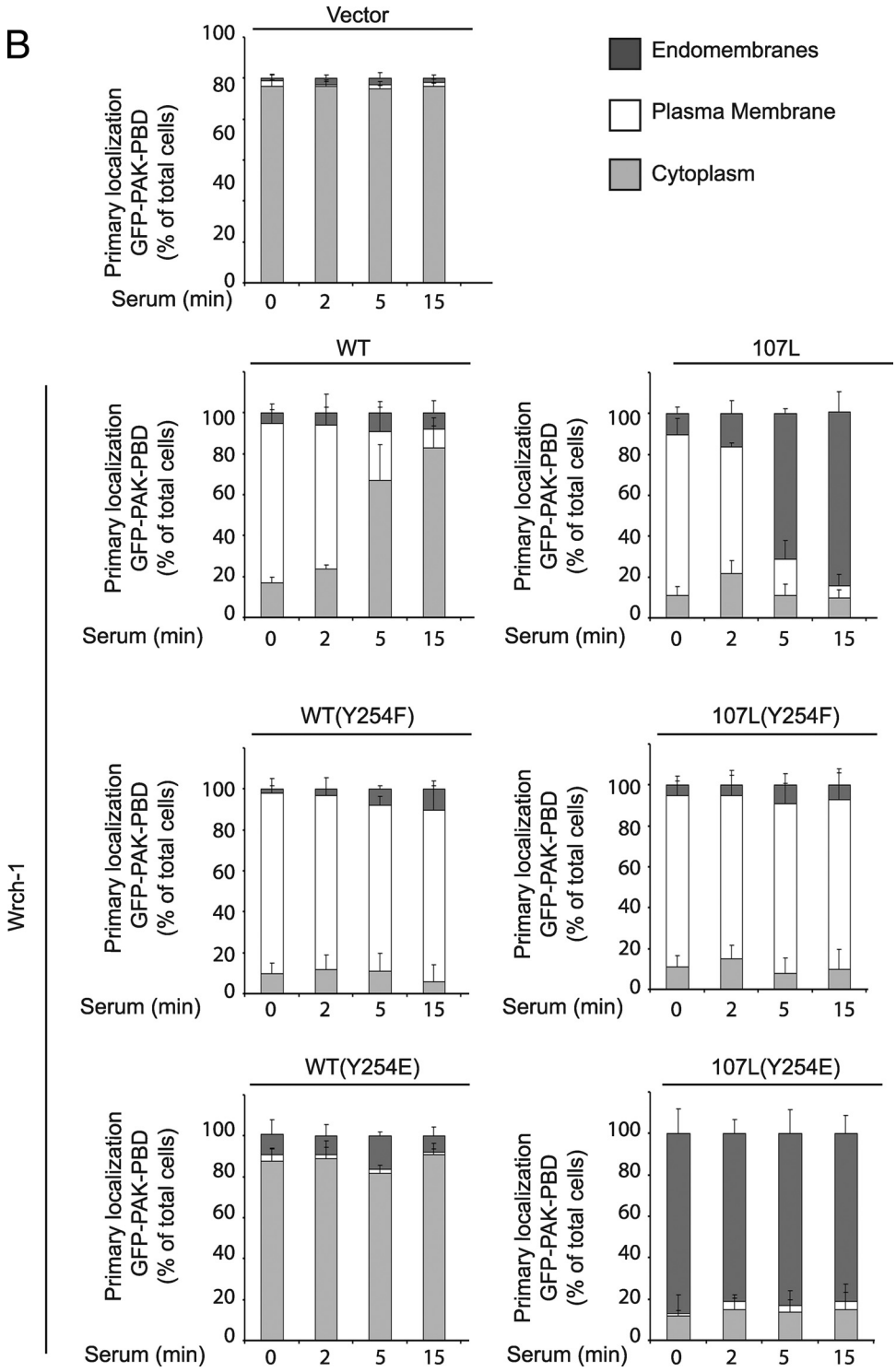

FIG. 6—*Continued*.

sufficient to abrogate C-terminal phosphorylation-mediated downregulation of Wrch-1 activity and to retain some Wrch-1 in an active conformation.

Finally, if phosphorylation of Wrch-1 at Y254 is sufficient for relocalization and subsequent downregulation of Wrch-1 activity and effector interaction, then a phospho-mimetic mutation should impair plasma membrane binding and instead confer constitutive internal localization and poor effector interaction. Consistent with this, a phospho-mimetic Y254E mutant was indeed largely excluded from the plasma membrane, localized to internal membranes, and failed to recruit PAK-PBD (Fig. 6A), which remained mostly cytosolic

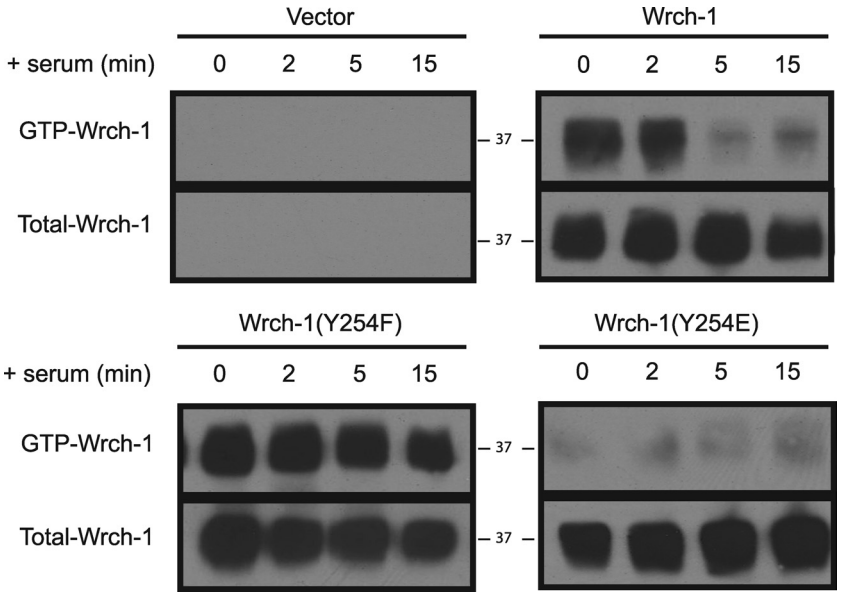

FIG. 7. Serum stimulation decreases active Wrch-1-GTP. To pull down active Wrch-1-GTP with GST-PAK-PBD, H1299 cells were transiently transfected with pCGN vector or vector encoding HA-tagged Wrch-1 proteins, then treated as for Fig. 6. Resulting cell lysates were incubated with GST-PAK fusion protein attached to glutathione-agarose beads. The Wrch-1/GST-PAK-PBD complex was collected by centrifugation, washed, eluted from the beads, and resolved by SDS-PAGE. Wrch-1 was detected by immunoblotting with anti-HA antibody. (Upper panels) Pull down followed by immunoblotting (GTP-Wrch-1); (lower panels) immunoblot of input total Wrch-1 available for pull down in the samples.

(>83% [Fig. 6B]) unless Wrch-1(Y254E) was constitutively GTP bound by virtue of a concurrent 107L mutation, in which case it was able to recruit PAK-PBD to its internal membrane location (Fig. 6A and B). These results also corroborate our evidence suggesting that localization regulates Wrch-1 GTP-binding status and that effector interaction of tyrosine-phosphorylated Wrch-1 is impaired as a consequence of decreased GTP binding rather than of decreased access to effector pools.

In order to confirm these results biochemically, we performed pull-down assays to detect the effects of serum stimulation on active Wrch-1-GTP under conditions where subcellular localization is irrelevant. PAK-PBD binds preferentially to active, GTP-bound Wrch-1 whether tagged by GFP (Fig. 6) or by GST (Fig. 7). We therefore transfected H1299 cells with empty vector or vectors encoding HA-tagged Wrch-1 proteins and treated them exactly as for the GFP-PAK-PBD localization assays depicted in Fig. 6. Cells were then lysed and incubated for 1 h with either purified GST or purified GST-PAK-PBD coupled to glutathione-agarose beads. The resulting protein complex was pulled down by centrifugation, washed, eluted off the beads, resolved by SDS-PAGE, and immunoblotted for the active, GTP-bound HA-Wrch-1 that was pulled down by interaction with GST-PAK-PBD. GST alone pulled down no Wrch-1 (data not shown), nor did GST-PAK-PBD pull down anything detectable by anti-HA antibody in the absence of HA-Wrch-1 (Fig. 7, vector panel). As predicted by the recruitment results from Fig. 6, GST-PAK-PBD pulled down Wrch-1 quite strongly under conditions when Wrch-1 localizes to the plasma membrane (Fig. 7, WT and Y254F, 0 min). In contrast, GST-PAK-PBD pulled down almost none of the Wrch-1 expected to be excluded from the plasma membrane (WT, 15 min; all Y254E) (Fig. 7). Similarly, by 5 min of serum stimulation, GST-PAK-PBD still pulled down Y254F robustly, but the amount of WT Wrch-1 pulled down was dramatically decreased (Fig. 7), consistent with the loss of WT but not Y254F from the plasma membrane. Taken together, these results support that serum stimulation of phosphorylation of Wrch-1 at Y254 causes its translocation from the plasma membrane and that the internal membrane pool is inactive.

Serum-stimulated tyrosine phosphorylation and relocalization of Wrch-1 decreases its activation of downstream effectors in a Y254-dependent manner. The decrease in Wrch-1/PAK effector interaction seen upon serum-stimulated tyrosine phosphorylation and subsequent relocalization of Wrch-1 strongly implies that Wrch-1 activates this effector preferentially at the plasma membrane and that loss from this compartment impairs effector activation. PAK-PBD contains the GTPase-binding domain but is not the full-length PAK kinase effector of Wrch-1. Further, active Wrch-1 has been shown to stimulate autophosphorylation and activation of both the serine/threonine kinase PAK1 (44) and the nonreceptor tyrosine kinase Pyk2 (34). Therefore, we tested whether the same conditions that relocalized Wrch-1 and decreased active Wrch-1-GTP also altered activation of these two kinases that are immediate downstream effectors of Wrch-1. Immunoblot analysis showed robust phosphorylation at the autophosphorylation sites of both PAK (Fig. 8A) and Pyk2 (Fig. 8B) in the presence of Wrch-1 WT or Y254F but not Y254E prior to serum stimulation, consistent with their interactions with PAK-PBD. Indeed, levels of both phospho-PAK1 and -Pyk2 were markedly decreased in the presence of phospho-mimetic Wrch-1(Y254E) and did not change regardless of serum stimulation. Similarly, serum decreased autophosphorylation of PAK and PYK2 in the presence of WT Wrch-1 (~95% and 38% decrease, respectively), whereas Y254F conferred resistance to the decrease (~15% and no decrease, respectively) (Fig. 8). Cells expressing

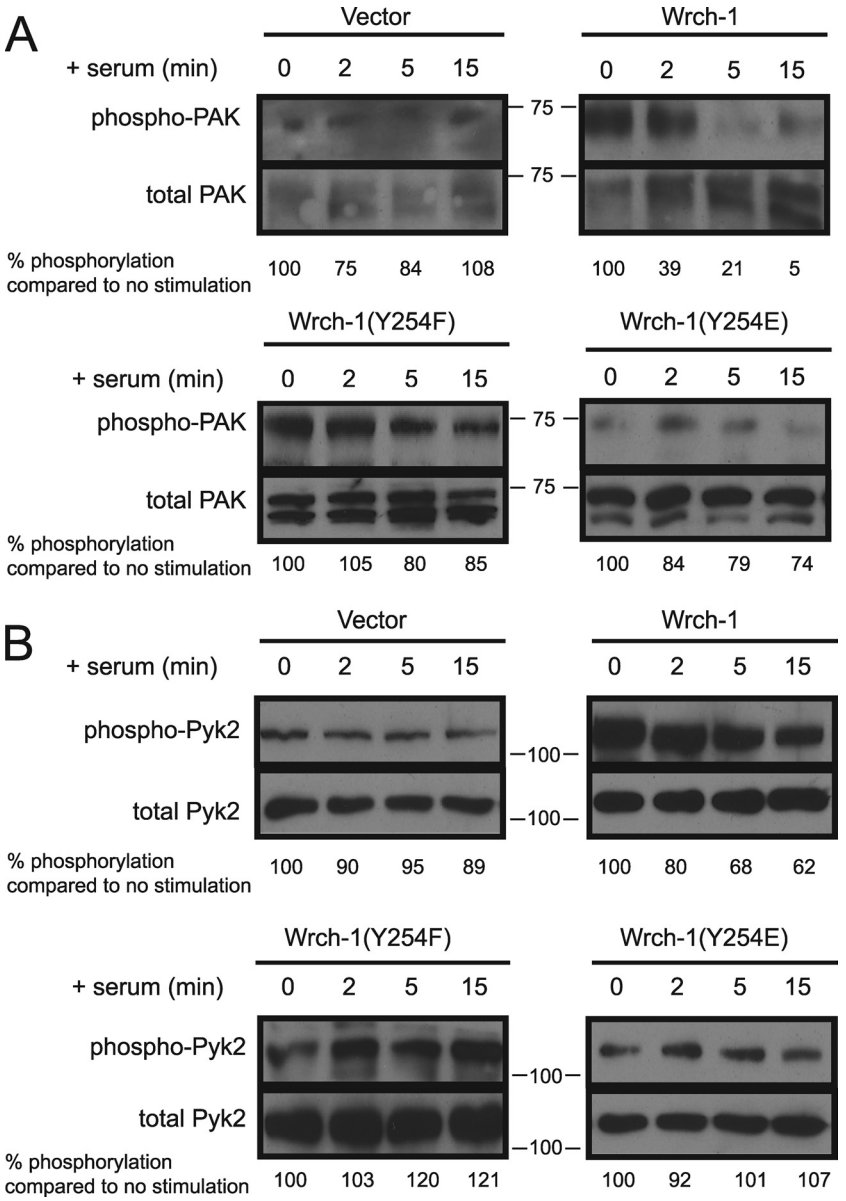

FIG. 8. Serum-stimulated Wrch-1 tyrosine phosphorylation results in decreased Wrch-1 effector activation. (A) Serum stimulation results in decreased autophosphorylation of PAK1. Cells treated as described for Fig. 7 were probed for total PAK with an anti-PAK1/2/3 antibody or for active phospho-PAK with an anti-phospho(Thr423)-PAK1/phospho(Thr402)-Pak2 antibody. (B) Serum stimulation results in decreased Pyk2 autophosphorylation. Cells treated as for panel A were probed for total Pyk2 with anti-Pyk2 antibody and for phospho-Pyk2 with anti-phospho(Tyr402)-Pyk2 antibody. Densitometry was performed using ImageJ software. Phosphoprotein was normalized to total protein in each lane, and the percent phosphorylation shown represents phosphorylation compared to no (0 min) serum stimulation.

empty vector were resistant to such a decrease (Fig. 8). Taken together, our results are consistent with a model in which C-terminal tyrosine phosphorylation downregulates Wrch-1 activity, perhaps by decreasing interactions with a putative GEF or by bringing it into proximity with an internally localized GAP.

DISCUSSION

We have shown here that the atypical Rho family small GTPase Wrch-1 undergoes serum-stimulated Src-mediated tyrosine phosphorylation at residue Y254 in its C-terminal membrane targeting domain, a modification that dynamically alters its subcellular localization by promoting relocalization from the plasma membrane. We also showed that Y254 is the major site of phosphorylation and that mutation of Y254 to a non-phosphorylatable phenylalanine residue (Y254F) prevents relocalization of the plasma membrane pool, remains GTP bound, enhances recruitment of the GTPase-binding domain of the Wrch-1 effector PAK1 (PAK-PBD), and enhances Wrch-1-mediated effects on growth transformation and on epithelial morphogenesis in 3D culture. In contrast, both serum-stimulated WT Wrch-1 and phospho-mimetic Y254E are re-

stricted from the plasma membrane and are inactive, failing to recruit PAK-PBD or to activate the effector kinases PAK or Pyk2 unless mutationally activated to be GAP insensitive (Q107L) and therefore constitutively active. These results indicate that C-terminal tyrosine phosphorylation of Wrch-1 may be important for downregulation of its biological activities, and they provide evidence supporting a potential mechanism whereby loss of interaction with an unidentified plasma membrane-associated GEF or increased association with a novel endosomally localized Wrch-1 GAP turns off previously active Wrch-1-GTP.

Our results demonstrate that Wrch-1 is GTP bound and active at the plasma membrane, where it is capable of recruiting and activating downstream effectors such as PAK1 and Pyk2. In contrast, serum-stimulated, Src-dependent phosphorylation at Y254 results in loss of Wrch-1 from the plasma membrane and loss of activity, and the endosomal pool of Wrch-1 is inactive. That the constutively active, GAP-insensitive mutant Q107L remains capable of effector recruitment at endosomes even when targeted there by the C-terminal phospho-mimetic mutation Y254E indicates that phosphorylation-mediated relocalization does not decrease Wrch-1 effector activation simply by removing Wrch-1 from its relevant effectors, at least some of which are still present at endosomes. For example, the well-validated Wrch-1 effector PAK is not only present at endosomes but is recruited and activated preferentially there by Chp/Wrch-2 in response to tumor necrosis factor alpha (12). Instead, our results support a model in which loss of Wrch-1 from the plasma membrane pool upon C-terminal phosphorylation at Y254 actively promotes its deactivation, since the major location is then the endosomal pool, which is not active.

Relocalization from the plasma membrane may decrease interaction of Wrch-1 with a plasma membrane-localized GEF or enhance its interaction with an endosomally localized GAP. No GEFs or GAPs for Wrch-1 have been identified, although both are presumed to exist. It is possible that known Cdc42-activating GEFs such as the FGD family, Tuba, Asef, or Intersectin may promote Wrch-1 activity at the plasma membrane, although these tend to be activated by growth factors or serum, whereas Wrch-1 is inactivated upon serum stimulation. On the other hand, endosomal GAPs, such as p50RhoGAP (40) and ARAP1 (14, 49), have been identified for other Rho family GTPases, but it is not known whether they promote GTP hydrolysis on Wrch-1. Other mechanisms of endosomal translocation and subsequent deactivation are well-documented for receptor tyrosine kinases (RTKs). Upon autophosphorylation and activation following growth factor stimulation, many RTKs become ubiquitinated and undergo relocalization to the endosomal compartment, which attenuates their signaling (15, 16). Additionally, ubiquitination of the small GTPase H-Ras has been shown to promote its endosomal trafficking and thereby to attenuate its signaling through the Raf-MEK-extracellular signal-regulated kinase pathway (21). We have observed that Wrch-1 is also ubiquitinated but that this modification does not appear to grossly alter its localization (data not shown). Thus, our present data best support the model of C-terminal phosphorylation of Wrch-1 leading to its downregulation by decreasing proximity to one or more GEFs and/or enhancing proximity to one or more GAPs.

Accumulating evidence suggests that phosphorylation of the C-terminal membrane targeting domains on small GTPases combines with other sequences and posttranslational modifications to dynamically regulate the localization and function of these proteins. To date, all the phosphorylation sites so identified, whether in Ras or Rho GTPases, have been Ser/Thr residues just upstream of C-terminal farnesyl or geranylgeranyl isoprenoid modifications (6, 17, 25–28, 30, 33). However, the atypical Rho family GTPase Wrch-1 is neither modified by isoprenylation nor possesses cognate serine or threonine residues. Instead, our study provides the first report of direct regulation of Rho family GTPase subcellular localization and function by tyrosine phosphorylation of its membrane targeting domain. Although Cdc42 has been reported to be tyrosine phosphorylated by Src upon epidermal growth factor stimulation (45), this phosphorylation occurs at residue Y64 within the switch II region rather than in the Cdc42 C terminus, which lacks any tyrosine residue. Whether tyrosine phosphorylation of Cdc42 alters its subcellular localization was not explored, but it was reported to promote binding of Cdc42 to RhoGDI. However, Wrch-1 is not modified by an isoprenoid, a feature required for binding of RhoGDI (42), and does not interact with RhoGDI (4). Thus, tyrosine phosphorylation of Wrch-1 and Cdc42 occurs on distinct domains and has distinct consequences. Chp/Wrch-2, the closest relative of Wrch-1, is also palmitoylated and unprenylated (12), but, like Cdc42, Chp lacks a tyrosine residue near its C terminus. This is perhaps not entirely surprising, as many closely related isoforms of small GTPases differ from each other mostly in their membrane targeting domains, possibly to provide signaling diversity.

Wrch-1 tyrosine phosphorylation at Y254 occurs only two residues away from the critical palmitoylation site C256, raising the question of whether one modification sterically hinders the other. It is clear that tyrosine phosphorylation of Wrch-1 does not require prior palmitoylation, as even a nonpalmitoylatable Cys→Ser mutant becomes tyrosine phosphorylated upon serum stimulation (data not shown). Indirect evidence suggests that this phosphorylation can occur simultaneously with palmitoylation: phosphorylation results in an increased association with internal membranes, whereas an inability to be palmitoylated results in a complete lack of Wrch-1 membrane association (5). Taken together, these results suggest that tyrosine phosphorylation normally occurs on palmitoylated Wrch-1.

We have shown here that Wrch-1 tyrosine phosphorylation requires Src and that Src can directly phosphorylate Wrch-1 *in vitro*. Although Y254, the major residue for tyrosine phosphorylation, does not occur in the context of a known Src kinase consensus site, and algorithms such as NetPhos or ScanSite do not predict its phosphorylation by Src, there is currently no reliable predictor of whether a given protein is in fact a substrate for Src *in vivo*. Even enolase, a commonly used positive control for Src phosphorylation, does not contain a known Src consensus site. However, many tyrosine kinases require phosphorylation themselves in order to be active, and this activating or priming phosphorylation step may be accomplished in *cis* or in *trans*. Therefore, other than direct phosphorylation of Wrch-1 by Src, another possibility is that Src activity promotes binding to Wrch-1 and/or activation of another tyrosine kinase that can phosphorylate it. Whether Wrch-1 is a direct or an indirect downstream target of Src-mediated phosphorylation in cells remains to be determined.

Recent studies have suggested several context-dependent functional connections between Wrch-1 and Src that are likely to be pertinent regardless of whether the connection is direct or indirect. In osteoclasts, Wrch-1 colocalizes with Src in podosomes, and increased Wrch-1 activity perturbs the podosome belt (31). Thus, Src-mediated tyrosine phosphorylation of Wrch-1 could contribute to the dynamic regulation of podosome formation and assembly. In addition, Wrch-1 negatively regulates macrophage colony-stimulating factor (M-CSF)-stimulated osteoclast migration (9), and Src has recently been shown to be activated in osteoclasts downstream of M-CSF stimulation (48). Therefore, M-CSF may promote osteoclast migration by activating Src, to then downregulate Wrch-1 through tyrosine phosphorylation on Y254. In contrast to osteoclasts, where it decreases migration, Wrch-1 increases the migration of fibroblasts (13), where it is localized to focal adhesions (31) and regulates their assembly. Src and several of its substrates are major components of focal adhesions. In PAE cells, Wrch-1 but not Cdc42 requires Src to induce formation of filopodia (34), but it is unknown whether Src is required for an effect on Wrch-1 itself or on a downstream target not shared with Cdc42.

We have observed that Y254, the major site of Src-mediated tyrosine phosphorylation, negatively regulates Wrch-1-mediated anchorage-independent growth and epithelial cell morphogenesis, because mutation to the nonphosphorylatable Y254F conferred greater activity on Wrch-1 than the wild-type tyrosine residue. Thus, Src-mediated tyrosine phosphorylation at Y254 may normally act as a brake for Wrch-1 function. Although Src is often thought of simply as an oncogene that leads inexorably to cellular dedifferentiation, it is clear that it can exert dual functions. For example, Src serves dual functions during epithelial cell morphogenesis in *Drosophila melanogaster*, where it both antagonizes E-cadherin-mediated cell adhesion and simultaneously stimulates E-cadherin transcription (37). Similarly, if Src modulates Wrch-1 through opposing functions, then proper cycling between the phosphorylated and the unphosphorylated state of Wrch-1 is likely to be required for the correct final outcome, regardless of whether Wrch-1 is a direct or indirect target of Src kinase activity. It will certainly also be of interest to determine which phosphatase(s) contributes to restoration of the unphosphorylated state of Wrch-1.

Our observations identify important contributors to Wrch-1 regulation and lend further credence to the emerging paradigm that C-terminal phosphorylation of small GTPases may serve as a key mechanism to dynamically regulate their localization, activation, and function. Thus, further investigations into such phosphorylation events will be critical for a better understanding of the regulation of Rho GTPases.

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REFERENCES

1. **Adamson, P., C. J. Marshall, A. Hall, and P. A. Tilbrook.** 1992. Post-translational modifications of p21rho proteins. J. Biol. Chem. **267:**20033–20038.
2. **Aspenstrom, P., A. Ruusala, and D. Pacholsky.** 2007. Taking Rho GTPases to the next level: the cellular functions of atypical Rho GTPases. Exp. Cell Res. **313:**3673–3679.
3. **Bain, J., L. Plater, M. Elliott, N. Shpiro, C. J. Hastie, H. McLauchlan, I. Klevernic, J. S. Arthur, D. R. Alessi, and P. Cohen.** 2007. The selectivity of protein kinase inhibitors: a further update. Biochem. J. **408:**297–315.
4. **Berzat, A. C., D. C. Brady, J. J. Fiordalisi, and A. D. Cox.** 2006. Using inhibitors of prenylation to block localization and transforming activity. Methods Enzymol. **407:**575–597.
5. **Berzat, A. C., J. E. Buss, E. J. Chenette, C. A. Weinbaum, A. Shutes, C. J. Der, A. Minden, and A. D. Cox.** 2005. Transforming activity of the Rho family GTPase, Wrch-1, a Wnt-regulated Cdc42 homolog, is dependent on a novel carboxyl-terminal palmitoylation motif. J. Biol. Chem. **280:**33055–33065.
6. **Bivona, T. G., S. E. Quatela, B. O. Bodemann, I. M. Ahearn, M. J. Soskis, A. Mor, J. Miura, H. H. Wiener, L. Wright, S. G. Saba, D. Yim, A. Fein, I. Perez de Castro, C. Li, C. B. Thompson, A. D. Cox, and M. R. Philips.** 2006. PKC regulates a farnesyl-electrostatic switch on K-Ras that promotes its association with Bcl-XL on mitochondria and induces apoptosis. Mol. Cell **21:**481–493.
7. **Blake, R. A., M. A. Broome, X. Liu, J. Wu, M. Gishizky, L. Sun, and S. A. Courtneidge.** 2000. SU6656, a selective src family kinase inhibitor, used to probe growth factor signaling. Mol. Cell. Biol. **20:**9018–9027.
8. **Brady, D. C., J. K. Alan, J. P. Madigan, A. S. Fanning, and A. D. Cox.** 2009. The transforming Rho family GTPase Wrch-1 disrupts epithelial cell tight junctions and epithelial morphogenesis. Mol. Cell. Biol. **29:**1035–1049.
9. **Brazier, H., G. Pawlak, V. Vives, and A. Blangy.** 2009. The Rho GTPase Wrch1 regulates osteoclast precursor adhesion and migration. Int. J. Biochem. Cell Biol. **41:**1391–1401.
10. **Brazier, H., S. Stephens, S. Ory, P. Fort, N. Morrison, and A. Blangy.** 2006. Expression profile of RhoGTPases and RhoGEFs during RANKL-stimulated osteoclastogenesis: identification of essential genes in osteoclasts. J. Bone Miner. Res. **21:**1387–1398.
11. **Bryant, D. M., and K. E. Mostov.** 2008. From cells to organs: building polarized tissue. Nat. Rev. Mol. Cell Biol. **9:**887–901.
12. **Chenette, E. J., A. Abo, and C. J. Der.** 2005. Critical and distinct roles of amino- and carboxyl-terminal sequences in regulation of the biological activity of the Chp atypical Rho GTPase. J. Biol. Chem. **280:**13784–13792.
13. **Chuang, Y. Y., A. Valster, S. J. Coniglio, J. M. Backer, and M. Symons.** 2007. The atypical Rho family GTPase Wrch-1 regulates focal adhesion formation and cell migration. J. Cell Sci. **120:**1927–1934.
14. **Daniele, T., G. Di Tullio, M. Santoro, G. Turacchio, and M. A. De Matteis.** 2008. ARAP1 regulates EGF receptor trafficking and signalling. Traffic **9:**2221–2235.
15. **Dikic, I.** 2003. Mechanisms controlling EGF receptor endocytosis and degradation. Biochem. Soc. Trans. **31:**1178–1181.
16. **Dikic, I., and S. Giordano.** 2003. Negative receptor signalling. Curr. Opin. Cell Biol. **15:**128–135.
17. **Ellerbroek, S. M., K. Wennerberg, and K. Burridge.** 2003. Serine phosphorylation negatively regulates RhoA in vivo. J. Biol. Chem. **278:**19023–19031.
18. **Etienne-Manneville, S., and A. Hall.** 2002. Rho GTPases in cell biology. Nature **420:**629–635.
19. **Isomura, M., A. Kikuchi, N. Ohga, and Y. Takai.** 1991. Regulation of binding of rhoB p20 to membranes by its specific regulatory protein, GDP dissociation inhibitor. Oncogene **6:**119–124.
20. **Jaffe, A. B., and A. Hall.** 2005. Rho GTPases: biochemistry and biology. Annu. Rev. Cell Dev. Biol. **21:**247–269.
21. **Jura, N., E. Scotto-Lavino, A. Sobczyk, and D. Bar-Sagi.** 2006. Differential modification of Ras proteins by ubiquitination. Mol. Cell **21:**679–687.
22. **Kirikoshi, H., and M. Katoh.** 2002. Expression of WRCH1 in human cancer and down-regulation of WRCH1 by beta-estradiol in MCF-7 cells. Int. J. Oncol. **20:**777–783.
23. **Klinghoffer, R. A., C. Sachsenmaier, J. A. Cooper, and P. Soriano.** 1999. Src family kinases are required for integrin but not PDGFR signal transduction. EMBO J. **18:**2459–2471.
24. Reference deleted.
25. **Lang, P., F. Gesbert, M. Delespine-Carmagnat, R. Stancou, M. Pouchelet, and J. Bertoglio.** 1996. Protein kinase A phosphorylation of RhoA mediates the morphological and functional effects of cyclic AMP in cytotoxic lymphocytes. EMBO J. **15:**510–519.
26. **Lerosey, I., V. Pizon, A. Tavitian, and J. de Gunzburg.** 1991. The cAMP-dependent protein kinase phosphorylates the rap1 protein in vitro as well as in intact fibroblasts, but not the closely related rap2 protein. Biochem. Biophys. Res. Commun. **175:**430–436.
27. **Lim, K. H., D. C. Brady, D. F. Kashatus, B. B. Ancrile, C. J. Der, A. D. Cox, and C. M. Counter.** 9 November 2009, posting date. Aurora-A phosphoryl-

ates, activates, and relocalizes RalA. Mol. Cell. Biol. **30:**508–523. [Epub ahead of print.]
28. **Madigan, J. P., B. O. Bodemann, D. C. Brady, B. J. Dewer, P. J. Keller, M. Leitges, M. R. Philips, A. J. Ridley, C. J. Der, and A. D. Cox.** 2009. Regulation of Rnd3 localization and function by PKCa-mediated phosphorylation. Biochem. J. **424:**153–161.
29. **Mumby, S. M., C. Kleuss, and A. G. Gilman.** 1994. Receptor regulation of G-protein palmitoylation. Proc. Natl. Acad. Sci. U. S. A. **91:**2800–2804.
30. **Okada, S., E. Yamada, T. Saito, K. Ohshima, K. Hashimoto, M. Yamada, Y. Uehara, T. Tsuchiya, H. Shimizu, K. Tatei, T. Izumi, K. Yamauchi, S. Hisanaga, J. E. Pessin, and M. Mori.** 2008. CDK5-dependent phosphorylation of the Rho family GTPase TC10(alpha) regulates insulin-stimulated GLUT4 translocation. J. Biol. Chem. **283:**35455–35463.
31. **Ory, S., H. Brazier, and A. Blangy.** 2007. Identification of a bipartite focal adhesion localization signal in RhoU/Wrch-1, a Rho family GTPase that regulates cell adhesion and migration. Biol. Cell **99:**701–716.
32. **Quilliam, L. A., H. Mueller, B. P. Bohl, V. Prossnitz, L. A. Sklar, C. J. Der, and G. M. Bokoch.** 1991. Rap1A is a substrate for cyclic AMP-dependent protein kinase in human neutrophils. J. Immunol. **147:**1628–1635.
33. **Riento, K., N. Totty, P. Villalonga, R. Garg, R. Guasch, and A. J. Ridley.** 2005. RhoE function is regulated by ROCK I-mediated phosphorylation. EMBO J. **24:**1170–1180.
34. **Ruusala, A., and P. Aspenstrom.** 2008. The atypical Rho GTPase Wrch1 collaborates with the nonreceptor tyrosine kinases Pyk2 and Src in regulating cytoskeletal dynamics. Mol. Cell. Biol. **28:**1802–1814.
35. **Saras, J., P. Wollberg, and P. Aspenstrom.** 2004. Wrch1 is a GTPase-deficient Cdc42-like protein with unusual binding characteristics and cellular effects. Exp. Cell Res. **299:**356–369.
36. **Schiavone, D., S. Dewilde, F. Vallania, J. Turkson, F. Di Cunto, and V. Poli.** 2009. The RhoU/Wrch1 Rho GTPase gene is a common transcriptional target of both the gp130/STAT3 and Wnt-1 pathways. Biochem. J. **421:**283–292.
37. **Shindo, M., H. Wada, M. Kaido, M. Tateno, T. Aigaki, L. Tsuda, and S. Hayashi.** 2008. Dual function of Src in the maintenance of adherens junctions during tracheal epithelial morphogenesis. Development **135:**1355–1364.
38. **Shutes, A., A. C. Berzat, E. J. Chenette, A. D. Cox, and C. J. Der.** 2006. Biochemical analyses of the Wrch atypical Rho family GTPases. Methods Enzymol. **406:**11–26.
39. **Shutes, A., A. C. Berzat, A. D. Cox, and C. J. Der.** 2004. Atypical mechanism of regulation of the Wrch-1 Rho family small GTPase. Curr. Biol. **14:**2052–2056.
40. **Sirokmany, G., L. Szidonya, K. Kaldi, Z. Gaborik, E. Ligeti, and M. Geiszt.** 2006. Sec14 homology domain targets p50RhoGAP to endosomes and provides a link between Rab and Rho GTPases. J. Biol. Chem. **281:**6096–6105.
41. **Sorkin, A., and M. Von Zastrow.** 2002. Signal transduction and endocytosis: close encounters of many kinds. Nat. Rev. Mol. Cell Biol. **3:**600–614.
42. **Takai, Y., K. Kaibuchi, A. Kikuchi, and T. Sasaki.** 1995. Effects of prenyl modifications on interactions of small G proteins with regulators. Methods Enzymol. **250:**122–133.
43. **Taneyhill, L., and D. Pennica.** 2004. Identification of Wnt responsive genes using a murine mammary epithelial cell line model system. BMC Dev. Biol. **4:**6.
44. **Tao, W., D. Pennica, L. Xu, R. F. Kalejta, and A. J. Levine.** 2001. Wrch-1, a novel member of the Rho gene family that is regulated by Wnt-1. Genes Dev. **15:**1796–1807.
45. **Tu, S., W. J. Wu, J. Wang, and R. A. Cerione.** 2003. Epidermal growth factor-dependent regulation of Cdc42 is mediated by the Src tyrosine kinase. J. Biol. Chem. **278:**49293–49300.
46. **Williams, C. L.** 2003. The polybasic region of Ras and Rho family small GTPases: a regulator of protein interactions and membrane association and a site of nuclear localization signal sequences. Cell Signal. **15:**1071–1080.
47. **Wu, J. C., T. Y. Chen, C. T. Yu, S. J. Tsai, J. M. Hsu, M. J. Tang, C. K. Chou, W. J. Lin, C. J. Yuan, and C. Y. Huang.** 2005. Identification of V23RalA-Ser194 as a critical mediator for Aurora-A-induced cellular motility and transformation by small pool expression screening. J. Biol. Chem. **280:**9013–9022.
48. **Yano, A., S. Tsutsumi, S. Soga, M. J. Lee, J. Trepel, H. Osada, and L. Neckers.** 2008. Inhibition of Hsp90 activates osteoclast c-Src signaling and promotes growth of prostate carcinoma cells in bone. Proc. Natl. Acad. Sci. U. S. A. **105:**15541–15546.
49. **Yoon, H. Y., J. S. Lee, and P. A. Randazzo.** 2008. ARAP1 regulates endocytosis of EGFR. Traffic **9:**2236–2252.
50. **Yu, H., X. Li, G. S. Marchetto, R. Dy, D. Hunter, B. Calvo, T. L. Dawson, M. Wilm, R. J. Anderegg, L. M. Graves, and H. S. Earp.** 1996. Activation of a novel calcium-dependent protein-tyrosine kinase. Correlation with c-Jun N-terminal kinase but not mitogen-activated protein kinase activation. J. Biol. Chem. **271:**29993–29998.